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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
TITLE (and Subtitle) Plasmids Coding for Colonization Factor Antigens I and II, LT, and ST-A2 in Escherichia coli		5. TYPE OF REPORT & PERIOD COVERED 6. PERFORMING ORG. REPORT NUMBER
AUTHOR(s) Peter Echeverria, Jitvimol Seriwatana, David N. Taylor, Suchitra Changchawalit, Cyril J. Smyth, Jane Twohig, and Bernard Rowe		8. CONTRACT OR GRANT NUMBER(s)
PERFORMING ORGANIZATION NAME AND ADDRESS AFRIMS APO San Francisco, CA 96346-5000		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research & Dev. Comm. Ft. Detrick, Frederick, MD 21701		12. REPORT DATE 13. NUMBER OF PAGES 5
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) Walter Reed Army Institute of Research Washington, DC 20307-5100		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Colonization factor antigens I and II (CFA/I and CFA/II) are important in the pathogenesis of diarrhea in humans caused by some enterotoxigenic Escherichia coli (ETEC). Plasmid DNA from 16 CFA/I+ and five CFA/II+ ETEC were examined by Southern blot analysis with enterotoxin gene probes, and compared with plasmid DNA from derivatives of these ETEC that had lost the ability to produce these colonization factors. Among 16 CFA/I+ ETEC strains, loss of CFA/I was accompanied by the loss of a plasmid of between 34 and 68 megadaltons (Mdal) coding for ST-A2 in twelve strains, with the loss of a 60 Mdal plasmi

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coding LT and ST-A2 in one strain, or with deletions of a segment of DNA encoding for ST-A2 in three strains. Among five CFA/II+ ETEC strains, loss of CFA/II was associated with the loss of a plasmid of 75 Mdal coding for LT and ST-A2 in three strains, the loss of genes coding for LT and ST-A2 from a 68 Mdal plasmid in one strain, or with no discernible loss of a plasmid or DNA sequences coding for enterotoxins in the remaining strain. The loss of CFA/I and CFA/II production was associated with the loss of DNA sequences encoding for ST-A2 in 20 of 21 ETEC examined.

INFECTION AND IMMUNITY, Feb. 1986, p. 626–630
0019-9567/86/020626-05$02.00/0

Vol. 51, No. 2

Plasmids Coding for Colonization Factor Antigens I and II, Heat-Labile Enterotoxin, and Heat-Stable Enterotoxin A2 in *Escherichia coli*

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Received 12 July 1985/Accepted 14 October 1985

Colonization factor antigens I and II (CFA/I and CFA/II) are important in the pathogenesis of diarrhea in humans caused by some enterotoxigenic *Escherichia coli* (ETEC). Plasmid DNA from 16 CFA/I$^+$ and five CFA/II$^+$ ETEC were examined by Southern blot analysis with enterotoxin gene probes and were compared with plasmid DNA from derivatives of the same ETEC that had lost the ability to produce these colonization factors. Among the 16 CFA/I$^+$ ETEC strains, the loss of CFA/I was accompanied by the loss of a plasmid of between 34 and 68 megadaltons (MDa) coding for heat-stable enterotoxin A2 (ST-A2) in 12 strains, by the loss of a 60-MDa plasmid coding for heat-labile enterotoxin (LT) and ST-A2 in one strain, or by deletions of a segment of DNA encoding for ST-A2 in three strains. Among five CFA/II$^+$ ETEC strains, the loss of CFA/II was associated with the loss of a plasmid of 75 MDa coding for LT and ST-A2 in three strains, with the loss of genes coding for LT and ST-A2 from a 68-MDa plasmid in one strain, or with no discernible loss of a plasmid or DNA sequences coding for enterotoxins in the remaining strain. The loss of CFA/I and CFA/II production was associated with the loss of DNA sequences encoding for ST-A2 in 20 of 21 ETEC examined.

In certain strains of enterotoxigenic *Escherichia coli* (ETEC) isolated from humans with diarrhea, two different fimbrial antigens, colonization factor antigens I and II (CFA/I and CFA/II), have been identified (10–15). These fimbrial antigens enable *E. coli* to attach to the luminal surface of the small intestines. Both attachment and enterotoxin production are important in the pathogenesis of *E. coli* diarrhea (10). The ability of an *E. coli* to produce CFA/I or CFA/II is plasmid mediated (10, 22, 27, 28, 37–39). Genes coding for CFA/I and heat-stable (ST) enterotoxin production have been found on the same plasmid (29, 37, 39), whereas genes coding for CFA/II production have been associated with plasmids coding for both heat-labile (LT) and ST enterotoxins (28, 38). Two different restriction endonuclease fragments of plasmid DNA encoding for ST-A, detectable in a suckling mouse assay, have been cloned and sequenced (19, 26). Cloned gene probes coding for ST-A1, derived from an ETEC of porcine origin (19), and for ST-A2, from an ETEC of human origin (26), did not cross-hybridize under stringent hybridization conditions. Moreover, Moseley et al. (25) demonstrated that *E. coli* H10407 contains a plasmid coding for ST-A2 and CFA/I and another plasmid coding for LT and ST-A1. Smith et al. and Willshaw et al. (39, 42) have previously shown that plasmid DNA sequences coding for ST were closely linked to a region involved in CFA/I production. Two regions of the plasmid were required for the expression of CFA/I, and these two sites were separated by a length of DNA corresponding to a molecular size of 25 megadaltons (MDa) (39). Genes coding for ST-A2 were associated with one of these regions. Other investigators have also noted an association between CFA/I and genes coding for ST and between CFA/II and genes coding for LT and ST (12, 22, 27, 28, 31, 37–39, 42).

To determine the association of genes coding for enterotoxins and colonization factors, plasmid DNA isolated from CFA/I$^+$ and CFA/II$^+$ ETEC and derivatives of those same isolates that had lost colonization factors were examined by Southern blot analysis with enterotoxin gene probes (41).

MATERIALS AND METHODS

Bacterial strains. The 21 ETEC strains examined in this study were isolated from different sources in Thailand in 1982 to 1983 (4). Isolates were tested for LT in Y1 adrenal cells (34) and for ST in the suckling mouse assay (7) immediately before being examined for colonization factors. ETEC isolates were tested for mannose-resistant hemagglutination of human group A and bovine erythrocytes and for agglutination in specific antisera to CFA/I and CFA/II (15).

CFA$^-$ derivatives. None of 21 CFA$^+$ ETEC spontaneously lost the ability to produce CFA after repeated passage on CFA agar (13). To obtain CFA$^-$ derivatives of these strains, CFA$^+$ ETEC were grown in increasing concentrations of novobiocin (23); surviving *E. coli* were isolated on CFA agar (13). Individual colonies were initially screened for mannose-resistant hemagglutination, followed by agglutination in CFA/I- and CFA/II-specific antisera, and for enterotoxin production (7, 11, 15, 34). The CFA$^+$ strains and their CFA$^-$ derivatives were biotyped as described by Scotland et al. (36). The CFA/II$^+$ ETEC and their CFA$^-$ derivatives were also tested for the three distinct fimbrial antigens of CFA/II CS1, CS2, and CS3 by double immunodiffusion with monospecific antisera (40). Antibiotic susceptibilities of CFA$^+$ ETEC and their CFA$^-$ derivatives were determined by the disk diffusion method (1).

Plasmid DNA. Plasmid DNA was isolated from CFA$^+$ and

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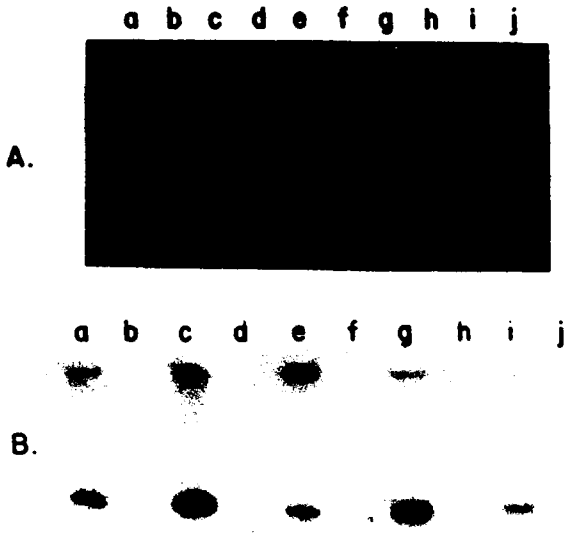

FIG. 1. Southern blot hybridization of plasmid DNA from CFA$^+$ and their CFA$^-$ *E. coli* derivatives. (A) Ethidium bromide-stained 0.7% agarose gel. Lanes: a, C529-1 LT$^+$ST$^+$ CFA/I$^+$ *E. coli*; b, C529-1P LT$^+$ST$^-$ CFA/I$^-$ *E. coli*; c, CC619G-1 LT$^+$ST$^+$ CFA/II$^+$ *E. coli*; d, CC619G-1P LT$^-$ ST$^-$ CFA/II$^-$ *E. coli*; e, CD663B-1 LT$^-$ ST$^+$ CFA/I$^+$ *E. coli*; f, CD663B-1P LT$^-$ ST$^-$ CFA/I$^-$ *E. coli*; g, C584-10 LT$^-$ ST$^+$ CFA/I$^+$ *E. coli*; h, C584-10P LT$^-$ ST$^-$ CFA/I$^-$ *E. coli*; i, C712-3 LT$^+$ST$^+$ CFA/I$^+$ *E. coli*; j, C712-3P LT$^+$ST$^-$ CFA/I$^-$ *E. coli*. (B) Autoradiograph of a Southern blot of the gel shown in panel A hybridized with the ST-A2 enterotoxin gene probe.

CFA$^-$ pairs of strains by the method of Birnboim and Doly (2), separated by electrophoresis on 0.7% agarose, stained with ethidium bromide, and photographed under UV illumination. DNA was transferred to nitrocellulose paper (BA-85; Schleicher & Schuell, Inc., Keene, N.H.) by the Southern technique (41) and examined for hybridization with the LT, ST-A1, and ST-A2 enterotoxin gene probes (25). Plasmid molecular weights were determined by their electrophoretic mobilities relative to plasmids of known molecular weights including plac, pPRC122, pR1, pRP4, pR6K, and pSa (3).

RESULTS

CFA/I$^+$ ETEC. The 16 CFA/I$^+$ ETEC belonging to four serogroups and three biotypes are shown in Table 1. The strains and CFA$^-$ derivatives that were of the same biotype and antibiogram were examined by Southern blot analysis with the LT, ST-A1, and ST-A2 gene probes. A Southern blot hybridization of plasmid DNA from CFA$^+$ and thus CFA$^-$ derivatives examined with the ST-A2 enterotoxin gene probe is shown in Fig. 1. As summarized in Table 2, the loss of CFA/I was associated with the loss of a plasmid between 34 and 68 MDa encoding for ST-A2 in 12 strains and with the loss of a 60-MDa plasmid coding for LT and ST-A2 in strain C625. The 39-MDa plasmid in strain C665-1P, but not in its parental strain, hybridized with the ST-A2 probe which suggested that the ST-A2 gene had transposed from the 68-MDa plasmid that was lost onto the 39-MDa plasmid. This strain was the only one of 16 CFA/I$^-$ derivatives that retained the ability to produce ST. In CFA/I$^+$ ETEC strains C54-9, C584-6, and C636-1 the loss of the ability of a plasmid to hybridize with the ST-A2 enterotoxin gene probe was associated with the loss of the ability to produce ST and CFA/I.

CFA/II$^+$ ETEC. The loss of a plasmid of 75 MDa encoding for LT and ST-A2 was associated with the loss of production of CFA/II antigens CS1 and CS3 in three isolates (Table 3). In isolate CF1D499-2 the loss of production of CFA/II antigens CS2 and CS3 was associated with the deletion of a segment of DNA of approximately 1 MDa that contained genes coding for LT and ST-A2. In isolate OAE108-04-6 of biotype F loss of the ability to produce CS2 and CS3 antigens was not associated with any apparent loss of plasmid DNA or of genes coding for enterotoxins. The loss of CFA/II antigens CS1, CS2, and CS3 was not associated with the loss of resistance to antibiotics in the latter instances.

DISCUSSION

In 20 of 21 CFA/I$^+$ and CFA/II$^+$ ETEC examined, the loss of CFA antigen production was associated with the loss of a plasmid or of a segment of DNA coding for ST-A2. In four CFA/I$^+$ ETEC the loss of CFA and ST-A2 occurred without any apparent change in plasmid molecular weight. This suggests that the region of the plasmid involved in CFA synthesis which was closely associated with genes for ST-A2 was small. The genes coding for ST were similar to the ST-A2 sequence described by DeWilde et al. (9) which is similar to the DNA sequence coding for ST cloned by Moseley et al. (26) and which was used as the ST-A2 probe in this study.

Although no linkage has been demonstrated between genes coding for ST and other colonization factors, it is remarkable that *E. coli* with colonization factors K99, 987P, and F41 isolated from animals with diarrhea produces ST alone (18). The close association between the genes coding for ST-A2 and CFA/I and between the genes coding for LT and ST-A2 and CFA/II suggests that the same environmental pressures that were involved in the selection of DNA sequences coding for ST-A2 may also have selected for colonization factors associated with ETEC isolated from humans. It would be of interest to determine whether DNA sequences coding for ST-A1 (15) or ST-A2 (9, 25, 26) are associated with genes coding for the colonization factors found on *E. coli* isolated from animals.

DNA sequences coding for ST-A1 were not associated with genes involved in either CFA/I or CFA/II synthesis in the strains examined. In strain S466-9 the ST-A1 genes

TABLE 1. Biotypes of 16 CFA/I$^+$ ETEC isolates

Test[a]	Result of test with indicated serogroup			
	O78 (n = 3)	O126 (n = 1)	O128 (n = 10)	O153 (n = 2)
Acid from:				
Adonitol	−	−	−	−
Dulcitol	−	−	+	−
Sorbitol	+	+	+	+
Xylose	+	+	+	+
Rhamnose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	−
Sorbose	−	−	−	−
Raffinose	+	+	+	−
Decarboxylase:				
Ornithine	+	+	+	+

[a] Tests were performed as described by Scotland et al. (36). Results are listed in the format used by Merson et al. (24). In addition to the results shown here, the 10 O128 ETEC produced acid for salicin.

TABLE 2. Characteristics and plasmid content of CFA/I⁺ ETEC and their CFA/I⁻ derivatives

Strain no.	Serogroup	Antibiotic resistance[a]	CFA/I	Toxin		Molecular wt of plasmids (10^6)
				LT	ST	
C54-9	O128	Cm, Sm, Su, Tc	+	+	+	75, 60[b,c]
C54-9P			−	+	−	75, 60[b]
279B-1	O128 (H12)	Cm, Sm, Su, Tc	+	+	+	80, 57,[b] 48, 34[c]
279B-1P			−	+	−	80, 57,[b] 48
S356-1	O128	Cm, Sm, Su, Tc	+	+	+	80, 65[c] 60,[b] 48
S356-1P			−	+	−	80, 60,[b] 48
C529-1	O128	None	+	+	+	90, 68,[c] 65[b]
C529-1P			−	+	−	90, 65[b]
CWBD529-1	O128	None	+	+	+	90, 68,[c] 65[b]
CWBD529-1P			−	+	−	90, 65[b]
C712-3	O128	Ap, Cm, Sm, Su, Tc	+	+	+	90, 68,[c] 65[b]
C712-3P			−	+	−	90, 65[b]
SCH885E	O128	Cm, Sm, Su, Tc	+	+	+	90, 62,[b] 50,[c] 34
SCH885E-P			−	+	−	90, 62,[b] 34
C665-1	O128	Cm, Sm, Su, Tc	+	+	+	90, 68,[c] 65,[b] 50, 39
C665-1P			−	+	+	90, 65,[b] 50, 39[c]
C625	O128	Cm, Sm, Su, Tc	+	+	+	75, 65, 60,[b,c] 52
C625P			−	−	−	75, 65, 52
C636-1	O128	Ap, Cm, Sm, Su, Tc	+	+	+	85, 60[b,c]
C636-1P			−	−	−	85, 60[b]
C41-4	O78 (H12)	Cm, Sm, Su, Tc	+	+	+	90, 68,[c] 64[b] 25
C41-4P			−	+	−	90, 64*, 25
C99-3	O78 (H12)	Ap, Cm, Tc	+	−	+	65, 56,[c] 42, 32[c]
C99-3P			−	−	−	65, 42
S902-1	O78	Ap, Cm, Tc	+	−	+	65, 56,[c] 43
S902-1P			−	−	−	65, 43
CD663B-1	O126 (H6)	Ap, Tc	+	−	+	75, 68,[c] 60, 42, 39
CD663B-1P			−	−	−	75, 60, 42
CWBC537-1	O153 (H24)	Ap, Km, Nm, Sm, Su, Tp	+	−	+	65,[c] 59, 56, 50, 42
CWBC537-1P			−	−	−	59, 56, 50, 42
C584-10	O153 (H45)	Ap, Sm, Su, Tc	+	−	+	65,[c] 57, 50
C584-10P			−	−	−	65, 57, 50

[a] Ap, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Su, sulfisoxazole (sulphafurazole); Tc, tetracycline; Tp, trimethoprim.

[b] Hybridized with the LT enterotoxin gene probe.

[c] Hybridized with the ST-A$_1$ enterotoxin gene probe; H type in parentheses.

located on a 27-MDa plasmid were not expressed or they produced levels of ST that were not detected in the suckling mouse assay. This isolate was similar to H10407P which also contains a plasmid coding for ST-A1 but does not produce ST (10, 26).

Although CFA/I has been reported by several authors in ETEC of serogroup O128 (4, 17, 21, 27, 31, 32, 35), the reported incidence of LT-ST or LT-producing strains within this serogroup has been rare (4–6, 16, 17, 35) compared with the frequencies of strains producing only ST (5, 8, 16, 17, 24, 30, 33, 35). Each of the 10 serogroup O128 strains reported herein and of the three reported earlier (4), all of which were Thai isolates, produced ST and LT. The production of LT as measured by the Y1 adrenal cell assay was confirmed in the present study by the demonstration of LT genes on plasmids by Southern blot analysis with an LT enterotoxin gene probe. The fermentation pattern of the O128 strains (Table 1) was identical to that reported for two O128ac:H12 strains that produced both LT and ST and that originated in Bangladesh (17). Thus, the LT-ST-producing, CFA/I⁺ group O128 ETEC reported to date appear to be of Asian origin.

Efforts to construct nontoxigenic *E. coli* capable of producing colonization factors for use as possible vaccine candidates are currently being pursued by a number of investigators (20, 37). It has been difficult to construct nontoxigenic CFA/I⁺ strains because the genes coding for

TABLE 3. Characteristics and plasmid content of CFA/II$^+$ ETEC and their CFA/II$^-$ derivatives

Strain no.	Serogroup	Biotype[a]	Antibiotic resistance[b]	Colonization factor antigens				Toxin		Molecular wt of plasmid (10^6)
				CFA/II	CS1	CS2	CS3	LT	ST	
CC619G-1	O6	A	None	+	+	−	+	+	+	75,[c,d] 42, 38, 27
CC619G-1P				−	−	−	−	−	−	42, 38, 27
Sp108D-1	O6 (H16)	A	None	+	+	−	+	+	+	75,[c,d] 42, 38, 27
Sp108D-1P				−	−	−	−	−	−	42, 38, 27
S466-9	O6 (H16)	A	None	+	+	−	+	+	+	75,[c,d] 57, 50, 27[e]
S466-9P				−	−	−	−	−	−	57, 50, 27[e]
CF1D499-2	O6	B	None	+	−	+	+	+	+	100,[c,d] 68,[c,d] 42
CF1D499-2P				−	−	−	−	+	+	100,[c,d] 67, 42
OAE108-04-6	O6	F	Ap, Cm, Tc	+	−	+	+	+	+	100,[c,d] 68, 42
OAE108-04-6P			Ap, Cm, Tc	−	−	−	−	+	+	100,[c,d] 68, 42

[a] Biotypes were determined by the method of Scotland et al (36).
[b] Ap, Ampicillin; Cm, chloramphenicol; Tc, tetracycline.
[c] Hybridized with LT enterotoxin gene probe.
[d] Hybridized with ST-A2 enterotoxin gene probe.
[e] Hybridized with ST-A1 enterotoxin gene probe, H type in parentheses.

ST, shown in this study to be ST-A2, and one region involved in CFA/I production are closely linked (37, 39, 42). The close association between genes coding for LT, ST-A2, and CFA/II may also be a problem in the construction in a nontoxigenic vaccine candidates capable of producing CFA/II. It may still be possible by DNA hybridization and cloning techniques to construct isolates which produce CFA antigens but which do not contain the DNA sequence that encodes for ST-A2.

ACKNOWLEDGMENTS

This study was supported in part by contract C6-181-81 from the World Health Organization. C.J.S. is supported by a grant from the Medical Research Council of Ireland.

We are grateful to S. Formal for providing *E. coli* H10407 (CFA/I$^+$) and H10407P (CFA/I$^-$) and to E. Boedeker for providing antisera to CFA/II. We thank Chittima Pitarangsi, Prani Ratarasarn, Orapan Chivoratanond, Vitaya Khungvalert, Pradith Nabumrung, Sawat Boonak, and Pornthip Siripraivan for technical assistance.

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